Cyclophilin A is required for efficient human cytomegalovirus DNA replication and reactivation

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INTRODUCTION

Human cytomegalovirus (HCMV) is a member of the subfamily Betaherpesvirinae. In its latent state, HCMV replication is restricted, although immune suppression of the host may result in viral reactivation. Therefore, immunologically immature and immunocompromised patients are extremely susceptible to HCMV disease (Horváth et al., 2000; Khoshnevis & Tyring, 2002; Vanciková & Dvorák, 2001). Despite several treatment options, the incidence of HCMV infection has increased as a result of organ allografting, immunosuppressive treatment and an increase in the number of human immunodeficiency virus (HIV)-infected patients (Casareale et al., 1989; Cheung & Teich, 1999; Lang et al., 2004; Potena et al., 2006; Söderberg-Nauclér et al., 1997).

After primary infection, HCMV becomes latent in cells of the myeloid lineage. Bone marrow-derived myeloid progenitor cells have been proposed to be the main HCMV latency reservoir (Bego & St Jeor, 2006; Hahn et al., 1998; Maciejewski et al., 1992; Riegler et al., 2000; Sinzger et al., 2008; Stanier et al., 1989; Taylor-Wiedeman et al., 1991, 1993; Zhuravskaya et al., 1997). The mechanisms of HCMV latency are not fully understood in terms of viral gene expression and the cellular factors involved. Pro-monocyte and monocyte cell lines have been used as in vitro model systems to study HCMV latency and reactivation (Auwerx, 1991; Delannoy et al., 1997; Ioudinkova et al., 2006; Numazaki et al., 1992). The human acute monocytic leukaemia cell line THP-1 is the most commonly used in vitro model for study of the mechanisms involved in macrophage differentiation and regulation of macrophage-specific genes. Furthermore, THP-1 cells have been shown repeatedly to be a model for HCMV latency, as the virus can persist in these cells and be reactivated following their differentiation. After treatment with phorbol esters, THP-1 cells differentiate into macrophage-like cells,
which mimic native monocyte-derived macrophages (Beisser et al., 2001; Mæß et al., 2010; Turtinen & Seufzer, 1994; Weinschenker et al., 1988). Substantial evidence has demonstrated that non-differentiated THP-1 cells do not support HCMV lytic infection unless they are differentiated by the addition of phorbol 12-myristate 13-acetate (PMA) or 12-O-tetradecanoylphorbol-13-acetate (Khaiboullina et al., 2004; Turtinen & Seufzer, 1994; Weinschenker et al., 1988). It has been suggested that immediate-early (IE) proteins IE1 and IE2, pp65 and pp150 are undetectable in undifferentiated THP-1 cells and that HCMV DNA polymerase UL54 and MIE protein levels are higher in differentiated than in undifferentiated THP-1 cells (Ioudinkova et al., 2006; Lee et al., 1999; Turtinen & Seufzer, 1994; Yee et al., 2007).

Cyclophilin A (CyPA) is the major intracellular receptor for the immunosuppressive drug cyclosporin A (CsA) (Handschumacher et al., 1984; Willenbrink et al., 1995). CsA is known to bind to and inhibit CyPA (Saini & Potash, 2006). It has been shown that CyPA promotes both the formation and the infectivity of virions of HIV-1 (Lammers et al., 2010; Leone et al., 2009; Li et al., 2009b; Solbak et al., 2010), as well as influenza A virus (Liu et al., 2009a). CyPA protein is also incorporated into vaccinia virus particles (Castro et al., 2003) and acts as a co-factor in hepatitis C infection (Chatterji et al., 2009; Fischer et al., 2010; Kaul et al., 2009; Liu et al., 2009b; Yang et al., 2008). Furthermore, during influenza A virus infection, over-expression of CyPA reduces the infectivity of the virus, whilst depletion of CyPA decreases virus replication (Liu et al., 2009a). CyPA has also been shown to be a factor in the regulation of murine cytomegalovirus (MCMV). Silencing CyPA in neural stem and progenitor cells (NSPCs) reduces virus yields by 50%, although MCMV replication in fibroblasts is unaffected (Kawasaki et al., 2007).

In this study, we determined that expression of the cellular protein CyPA is critical for efficient HCMV replication and reactivation in vitro. Silencing CyPA in human foreskin fibroblast (HF) cells delayed the expression of IE1/2 and the production of infectious virions, as well as significantly reducing viral DNA loads. Furthermore, CyPA-depleted THP-1 cells were not able to reactivate HCMV following differentiation. Taken together, these data suggest that HCMV requires a stable level of CyPA for successful virus replication, virion production and reactivation from infected cells.

**RESULTS**

**CyPA inhibition delays IE protein expression in HF cells**

In order to characterize the effects of the CyPA protein on a productive HCMV infection, we silenced CyPA protein expression in HF cells using a retroviral vector system. Cells were stably transduced with a CyPA or scrambled small interfering RNA (siRNA) containing retroviral vector to produce the cell lines HF_{siCyPA} and HF_{siScram}. The levels of CyPA transcript and protein were monitored by quantitative real-time PCR (qPCR) and Western blotting, respectively. A significant decrease in CyPA transcript expression (Fig. 1a) accompanied by the absence of CyPA protein (Fig. 1b) was observed in HF_{siCyPA} cells compared with HF (P<0.05) or HF_{siScram} (P<0.05) cells. CsA is an immunosuppressant drug known to bind and inhibit CyPA. HF cells were treated with CsA 30 min prior to infection, and CsA was maintained throughout infection. Protein samples were collected from 1 to 20 days post-infection (p.i.) to study CyPA and viral IE1/2 protein expression by Western blotting (Fig. 2a–c). Interestingly, IE1/2 protein expression was very low in HF + CsA cells until 5 days p.i., and was not detected in HF_{siCyPA} cells until 10 days p.i., compared with the normal HF and HF_{siScram} cells in which IE1/2 protein expression was...
detected as early as 1 day p.i. (Fig. 2a–d). In order to
determine whether IE1/2 protein expression could be
recovered in the absence of CsA, we infected HF cells in the
presence of CsA for 1 day and then removed CsA by washing
the cells and changing the medium. After CsA was removed
from the medium, infected HF cells were harvested for protein
extraction at 3–20 days p.i. and analysed by Western blotting
(Fig. 2e). IE1/2 protein expression was minimal in HF cells
from day 1 to 5 p.i. in presence of CsA; however, when CsA
was removed from the medium, IE1/2 expression was greatly
increased and its level was similar to that detected in normal
HF cells. These results suggested that the CyPA protein may be
involved in regulating IE1/2 protein expression.

**Inhibiting CyPA reduces HCMV lytic replication in
HF cells**

We further characterized the effects of CyPA on a lytic
HCMV infection by monitoring the formation of virus
plaques and changes in viral DNA loads in normal HF,
HFsiScram, HFsiCyPA and HF + CsA cells. A mixture of lysed
cells and cell supernatants from infected cells were collected
from 1 to 20 days p.i. and tested by plaque assay. HF,
HFsiScram, HFsiCyPA and HF + CsA cells showed an increase in
the production of virions over time (Fig. 3a, b). Interestingly,
production of infectious viruses was significantly reduced in
infected HF + CsA cells until 5 days p.i. (P<0.05 compared
with HF cells; Fig. 3a) and significantly delayed in HFsiCyPA
cells (P<0.05 compared with HFsiScram; Fig. 3b), as plaques
were not detected until 10 days p.i. These data indicated that
inhibiting/depleting CyPA significantly reduced/delayed the
production of infectious virions in HF cells, and is consistent
with the delay in IE1/2 protein expression observed in Fig. 2.

As CyPA is known to be an essential co-factor in the
replication of HIV-1 DNA (Braaten et al., 1996), we
studied its possible effect on HCMV replication. DNA was
isolated from HF, HF + CsA, HFsiScram and HFsiCyPA cells
from 1 to 20 days p.i. and real-time PCR was performed
to determine any changes in viral DNA load over time
(normalized against endogenous cellular DNA, using
the primers listed in Table S1, available in JGV Online).
HFsiScram cells produced significantly higher levels of viral
DNA over time compared with HFsiCyPA cells at 3–20 days
p.i. (P<0.05; Fig. 4). HF cells also produced significantly
higher viral DNA loads from 3 to 20 days p.i. compared
with HF + CsA cells (P<0.05; Fig. 4). Furthermore, we
observed only marginal levels of viral DNA in HF cells
-treated with CsA at each time point.

**Silencing CyPA in THP-1 cells prevents
expression of lytic viral genes**

CyPA protein expression was silenced in THP-1 cells using
a retroviral vector as described above and the resultant cell
line was termed THP-1siCyPA. A control THP-1 cell line expressing scrambled siRNA (THP-1siScram) was also generated. Depletion of CyPA transcript and protein was monitored by qRT-PCR and Western blotting, respectively (Fig. 5a, b). RNA was isolated from infected THP-1, THP-1siScram, THP-1siCyPA and THP-1 + CsA cells over a 20-day time course, as well as at two time points after cellular differentiation (5 and 10 days after PMA treatment on day 10 p.i.; Fig. 6a). The presence of hallmark lytic or latent viral transcripts was determined by RT-PCR. During a lytic infection, viral genes are temporally expressed, starting with IE genes, such as UL123, followed by early genes and lastly late genes, such as UL82. UL138 and UL81-82ast (which encodes the latency-associated protein LUNA) are expressed during both a lytic and latent cycle; however, these two genes can be considered markers of latency when they are expressed in the absence of lytic gene expression and virus replication. Similarly, it has been reported that IE genes are detected at early time points during the establishment of latent infection (Lee et al., 1999). UL123 was detected in our THP-1 and THP-1siScram cells for up to 5 days p.i., but not at 10 or 20 days p.i. (Fig. 6b, d). Interestingly, UL123 was only detected at low levels at 1 day p.i. in THP-1 + CsA cells, and was not detected at any time point in THP-1siCyPA cells (Fig. 6c, e). Following treatment of the cells with PMA (resulting in differentiation), UL123 expression was recovered in THP-1, THP-1siScram and THP-1 + CsA cells, whereas it was not detected in THP-1siCyPA cells. The late gene UL82 was not detected in THP-1, THP-1siScram or THP-1siCyPA cells prior to

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**Fig. 3.** Inhibition of CyPA expression in HF cells delays the production of infectious virions. Supernatant and cell slurry were collected from infected HF and HF + CsA cells (a) and HFsiScram and HFsiCyPA cells (b) over a 20-day time course. The mean viral titre for each sample was determined using a standard plaque assay. The mean ± SD of three separate experiments is shown. P values were calculated using Student’s t-test: HF versus HF + CsA cells, P < 0.05; HFsiScram versus HFsiCyPA cells, P < 0.05.

**Fig. 4.** Inhibition of CyPA protein expression reduces viral DNA load over time in HF cells. Total DNA was isolated from infected HF, HFsiScram, HFsiCyPA and HF + CsA cells over a 20-day time course. Real-time PCR was used to test for the accumulation of viral DNA. All samples were normalized to endogenous cellular controls. All primers are listed in Table S1. ***, P < 0.05 for HF + CsA or HFsiCyPA cells versus HF or HFsiScram cells, respectively. The mean ± SD of three separate experiments is shown.
differentiation; however, UL82 was detected in THP-1 cells treated with CsA at all time points p.i. (Fig. 6b–e), suggesting that THP-1 cells treated with CsA do not enter viral latency. Following cellular differentiation, UL82 expression was recovered in THP-1 and THP-1siCyPA cells but not in THP-1siScram cells. Low levels of the latency-associated gene UL138 were detected in both THP-1 and THP-1siCyPA cells from 3 to 20 days p.i. but not after cellular differentiation (Fig. 6b–e). In THP-1siCyPA cells, UL138 was not detected until 5 days p.i. and was maintained at low levels until 20 days p.i.; expression was not increased following treatment with PMA (Fig. 6c). Moreover, low levels of UL138 were detected in THP-1 + CsA cells as early as 1 day p.i. and maintained throughout the 20-day time course, but were not detected after treatment with PMA (Fig. 6e). Lastly, UL81-82ast encoding the LUNA protein was detected throughout infection for each cell type, both prior to and following PMA-induced cellular differentiation (Fig. 6b–e). Taken together, these data suggest that HCMV establishes a latent infection in THP-1 and THP-1siScram cells and can be reactivated following cellular differentiation with PMA. Interestingly, although infected THP-1siCyPA cells established a non-productive infection, characterized by the lack of lytic gene expression and the presence of latency-associated genes, we did not observe lytic gene expression following differentiation, suggesting a lack of viral reactivation. Overall, our results indicated that silencing of CyPA prevents viral reactivation.

**Silencing of CyPA prevents the expression of the IE proteins in THP-1 cells**

Protein samples were isolated from mock-infected and infected THP-1, THP-1siScram, THP-1siCyPA and THP-1 + CsA cells from 1 to 20 days p.i. to monitor changes in CyPA and IE1/2 protein expression. Differentiation of THP-1 cells was induced at 10 days p.i. by the addition of PMA to the medium. Following differentiation, two additional time points at 5 and 10 days post-differentiation were also analysed by Western blotting (Fig. 7a). CyPA depletion was maintained over time in THP-1siCyPA cells before their differentiation, and slightly higher levels were detected after differentiation (Fig. 7c). Interestingly, in THP-1, THP-1siScram and THP-1 + CsA cells, a decrease in CyPA protein expression following PMA-induced differentiation was noted (Fig. 7b, d). IE1/2 protein expression was below the level of detection in THP-1siCyPA and THP-1 + CsA cells at all time points, unlike in THP-1 and THP-1siScram cells in which IE1/2 proteins were detected until 5 days p.i. and following PMA-induced differentiation (Fig. 7b–e). In order to confirm that IE1/2 protein expression could be recovered following the removal of CsA from the medium, CsA was removed at 1 day p.i. in a separate experiment and IE1/2 protein expression was monitored (Fig. 7e). Consistent with our HF data, IE1/2 protein expression was successfully recovered following removal of CsA from the medium to give a similar pattern to that observed for THP-1 and THP-1siScram before and after PMA-induced differentiation.

**CyPA protein inhibition prevents virus reactivation from infected THP-1 cells**

Real-time PCR was performed on DNA isolated from infected THP-1, THP-1siScram, THP-1siCyPA and THP-1 + CsA cells from 1 to 20 days p.i., before and after differentiation, as described for HF cells. As expected for a latent infection, viral DNA load decreased over time in infected THP-1, THP-1siScram and THP-1siCyPA cells (Fig. 8). Interestingly, we observed a low level of increase in viral DNA loads in THP-1 + CsA cells at each time point when compared with untreated THP-1 cells pre-differentiation (P < 0.05). No significant difference (P > 0.05) between THP-1 and THP-1 + CsA cells was observed in viral DNA loads at 5 days after PMA-induced differentiation; however, at 10 days after differentiation, THP-1 cells harboured significantly higher viral DNA loads compared with THP-1 + CsA cells.
cells (P<0.05). Furthermore, no significant difference was observed between THP-1siScram and THP-1siCyPA cells (P>0.05); however, there was a significant difference following PMA-induced differentiation (P<0.05) (Fig. 8). These results suggested that CyPA protein may be involved in the reactivation of HCMV from infected THP-1 cells.
DISCUSSION

Host-cell cyclophilins are essential for the efficient replication of a number of viruses, including human hepatitis virus (Nakagawa et al., 2005; Yang et al., 2008), HIV (Sokolskaja & Luban, 2006), Japanese encephalitis virus (Kambara et al., 2011) and varicella-zoster virus (Bose et al., 2003). In the herpesvirus family, CyPA appears to play a crucial role in both HCMV and MCMV replication. Kawasaki et al. (2007) showed that calcineurin/cyclophilin inhibitor, CsA and the cyclophilin-specific inhibitor NIM811 reduced MCMV replication by preventing IE gene expression in NSPCs. They also reported that silencing of CyPA in NSPCs was associated with a 50% inhibition of MCMV yield. As cyclophilins are required for virus replication, it has been suggested that they could be targeted for antiviral therapy. Importantly, CyPA can be sequestered by CsA and non-immunosuppressive derivatives thereof, such as SCY-635, NIM-811 and Debio-025 (Ma et al., 2006). For example, alisporivir (Debio-025), a non-immunosuppressive CsA derivative that selectively inhibits cyclophilins, is being developed as a potential oral treatment of hepatitis C virus infection (Watashi, 2010). Furthermore, clinical trials of Debio-025 given in combination with pegylated-interferon/ribavirin have demonstrated superior efficacy compared with standard-of-care treatment (Flisiak et al., 2008; Paeshuyse et al., 2006).

In this study, we determined that the inhibition/depletion of CyPA in lytically infected HF cells resulted in a significant delay and a decrease in the production of infectious virions over a 20-day period. We also observed that the reduction in the expression of CyPA delayed the expression of the IE1/2 proteins, and significantly reduced the overall viral DNA load over time in HFsiCyPA and HF + CsA cells compared with HF cells with normal CyPA expression. We observed a more drastic effect on IE1/2

Fig. 7. Inhibition of CyPA expression in THP-1 cells inhibits IE protein expression, but does not affect viral entry. (a) Diagram of the timeline of infection. Infected cells were collected from 1 to 20 days p.i. (dpi), as well as at 5 and 10 days post-differentiation induced by the addition of PMA to the medium at 10 days p.i. (5dp and 10dp, respectively). (b–e) Protein was isolated from infected THP-1siScram (b), THP-1siCyPA (c), THP-1 (d) and THP-1 + CsA (e) cells over a 20-day time course, as in (a). (f) THP-1 + CsA cells were infected for 1 day, and CsA was then removed from the medium and protein was extracted and analysed for IE1/2 protein expression. Western blotting was used to monitor the protein expression levels of CyPA, IE1/2 protein and β-actin as a loading control. Each Western blot was run in duplicate and a representative result is shown.
protein expression in cells where CyPA was silenced compared with HF+CsA cells. This difference in the inhibition of IE1/2 protein expression is probably due to the fact that the siRNA is stably expressed and is constantly knocking down the expression of CyPA, whereas the treatment with CsA is limited. Furthermore, we noted that IE1/2 protein expression could be recovered in HF+CsA cells if CsA was simply removed from the medium.

Although it is possible that silencing CyPA via siRNA or treatment with CsA could affect virus entry, we do not believe that this is the case. Similar levels of viral DNA in each cell type at 1 day p.i. suggested that the cells were successfully infected under each condition.

We also studied the possible role of CyPA in latently infected THP-1 cells. The use of THP-1 cells as a model for HCMV latency has been vastly explored (Beisser et al., 2001; Turtinen & Seufzer, 1994; Weinschenker et al., 1988). We confirmed that HCMV is capable of establishing a latent infection in these cells. First, we showed that lytic gene expression was lost by 5 days p.i. in infected THP-1 and THP-1siScram cells, and that latent gene expression was maintained. Furthermore, differentiation of the cells using PMA recovered the expression of lytic genes, implying virus reactivation. Additionally, viral DNA loads remained constant and low over time, and no infectious particles were detected in the cultures. To test whether low levels of reactivation occurred, supernatants from infected THP-1 cells were used to infect HF cells, and no viral plaques were detected (data not shown). However, HCMV DNA loads dramatically increased after THP-1 cells were differentiated using PMA, and virus reactivation occurred. Persistence of the viral DNA over time with limited gene expression and no detectable infectious viral particle release, coupled with a full reactivation phenotype after monocyte differentiation constitute the hallmarks of a true latent infection. Depletion/inhibition of CyPA in THP-1 cells did not induce spontaneous HCMV reactivation from latently infected THP-1 cells. UL123 was detected at 1 day p.i. in THP-1+CsA cells, and expression was recovered following differentiation, unlike in THP-1 and THP-1siScram cells in which UL123 could be detected for up to 5 days p.i. and again after cellular differentiation. Interestingly, although UL82 gene expression was only detected after differentiation in THP-1 and THP-1siScram cells, it was detected in THP-1+CsA throughout infection, and was shown to increase following differentiation of the cells. UL138 gene expression was detected in THP-1siCyPA cells until 5 days p.i., unlike in THP-1 and THP-1siScram cells in which UL138 was detected only until 3 days p.i. We were also able to detect UL138 in infected THP-1+CsA cells as early as 1 days p.i., which was expressed through 20 days p.i. Lastly, UL81-82ast was detected in each of the four cell types throughout the course of infection. Taken together, this is evidence not only that HCMV is capable of establishing a latent infection in THP-1 cells but also that silencing CyPA prevents the expression of lytic viral genes. Interestingly, we observed that inhibition of CyPA by treatment with CsA prevented the establishment of latency and, furthermore, the expression of UL123 was below detection. It is likely that CsA affects alters more than just CyPA, which would explain the different effects on HCMV infection observed when silencing CyPA using siRNA compared with inhibiting CyPA with CsA. IE1/2 protein expression was below the level of detection in THP-1siCyPA and THP-1+CsA cells at all time points, yet we were able to recover IE1/2 protein expression in THP-1+CsA cells when CsA was removed from the medium. Furthermore, we noticed significantly less viral DNA over time in infected THP-1 cells compared with THP-1siCyPA cells but not in THP-1siScram cells pre-differentiation. As expected, in THP-1siScram and THP-1 cells treated with PMA, viral DNA replication and reactivation was achieved post-differentiation. Remarkably, we did not observe an increase in viral DNA load in THP-1siCyPA cells following reactivation. The lack of reactivation corresponded with the lack of IE1/2 protein expression following differentiation in the infected
THP-1CsCyPA cells. Surprisingly, we also noted that THP-1 + CsA cells harboured higher DNA loads at each time point compared with untreated THP-1 cells before differentiation, and a reactivation phenotype was observed after PMA treatment. These results suggest that these cells may never establish a fully latent infection, or that CsA is not fully active to overcome CyPA once the monocytes differentiate. We are currently unable to explain the differences observed between THP-1CsCyPA cells and THP-1 cells treated with CsA. However, it is important to note that, whilst siRNA treatment is specific for CyPA, CsA effects may be pleotropic, partially explaining such differences.

Our results suggest that reducing CyPA expression inhibited or delayed the reactivation of HCMV in THP-1 cells. Alternatively, as CyPA has been shown to be incorporated into the virions of HIV and influenza virus (Li et al., 2009a; Liu et al., 2009a), it is possible that the lack of reactivation in differentiated THP-1CsCyPA cells was due to the fact that more CyPA is needed to form new infectious virions. We also observed a decrease in CyPA protein expression in THP-1, THP-1Cr and THP-1 + CsA cells after differentiation with PMA. Although, we cannot explain this phenomenon, it is possible that CyPA levels differ naturally between monocytes and macrophages. One study reported that differentiation of monocytes can suppress CyPA expression (Greben’ová et al., 2006; Obchoei et al., 2011; Song et al., 2004). Furthermore, we observed a slight increase in CyPA protein expression in THP-1CsCyPA cells following differentiation with PMA. It is likely that differentiation affects the RNA silencing pathways, possibly making them less efficient. It has been reported previously that siRNA and microRNAs can be affected by cellular differentiation, in which case, differentiation can either up- or downregulate the gene of interest (Coley et al., 2010; Suzuki et al., 2009).

We observed a non-concordant phenotype for THP-1 cells treated with CsA when compared with THP-1CsCyPA cells. We believe this is due to the fact that CsA does not act solely on CyPA but acts as a general immunosuppressant, and therefore may be altering other cellular functions, which may affect the ability of the virus to enter a truly latent state. For this reason, we feel that silencing of CyPA is a better way of analysing the true effects of CyPA on the life cycle of HCMV. These studies confirmed that CyPA is an important factor involved in the entire life cycle of HCMV – lytic infection, latency establishment and reactivation. CyPA could serve as a potential therapeutic target in preventing HCMV reactivation. CsA is a common immunosuppressive drug used during organ transplantations. However, CsA has also been associated with HCMV reactivation in vivo, probably as a response from overall immunosuppression (Hornef et al., 1995). A combined treatment in which immunosuppression is achieved by treatment with CsA and where CyPA is targeted directly using non-immunosuppressive CsA derivatives could potentially prevent transplant rejection and at the same time control HCMV reactivation. This study has highlighted a potential role for CyPA in the life cycle of HCMV and has shown that, when CyPA is inhibited or depleted, HCMV replication is compromised.

**METHODS**

**Cell culture and HCMV infections.** HCMV stocks were propagated in HF cells and maintained as frozen stocks. Viral titres were determined using standard plaque assays (Prichard et al., 1999). HF cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10 % FBS. THP-1 cells were maintained in RPMI 1640 supplemented with 10 % FBS. THP-1 cells were infected at an m.o.i. of 5 p.f.u. ml⁻¹ with a low-passage clinical isolate of HCMV, whilst HF cells were infected at an m.o.i. of 0.5. HF cells were incubated with virus for 1 h under normal growing conditions. After the incubation time, virus-containing medium was removed, the cells were washed twice with sterile PBS and fresh medium was added. THP-1 cells were infected by centrifugation with the virus at 3000 r.p.m. in a bench microfuge for 90 min at 37 °C. After centrifugation, THP-1 cells were washed three times with sterile PBS, resuspended in RPMI 1640 plus 10 % FBS and cultured under normal growing conditions. To induce differentiation of THP-1 cells, 50 ng PMA ml⁻¹ was added to the medium at 10 days p.i.

**Western blot analysis.** Cell lysis and protein solubilization were performed using Cel-Lytic M Mammalian Cell Lysis/Extraction Reagent (Sigma), according to the manufacturer’s recommendations. Proteins were quantified using an EZQ assay (Invitrogen). Samples containing 20 μg total protein were mixed with 4 × SDS-PAGE loading buffer [250 mM Tris/HCl (pH 7.0), 40 % glycerol, 0.8 ml β-mercaptoethanol, 5 % SDS and 0.005 % bromophenol blue], boiled for 5 min, cooled on ice and separated by SDS-PAGE (7.5 or 12 % acrylamide). For Western blot analysis, after semi-dry electrophoretic transfer of proteins onto PVDF membranes, the membranes were incubated with 5 % powdered milk in Tris-buffered saline/Tween buffer [10 mM Tris/HCl (pH 8.0), 150 mM NaCl and 0.05 % Tween 20] for 1 h to block non-specific protein interactions. The membranes were then probed with the following primary antibodies: rabbit anti-human CyPA (diluted 1 : 500; Abcam), mouse anti-IE1/2 (1 : 250, clone MAB810; Millipore) and mouse anti-human actin (1 : 10,000; Sigma) for a loading control. The secondary antibodies used were peroxidase-labelled goat anti-rabbit or anti-mouse IgG HRP (Southern Biotechnology Associates). Antibody-tagged protein bands were detected using a DAB Peroxidase Substrate kit (Vector).

**CyPA silencing and CsA treatment.** CyPA protein expression was silenced in HF or THP-1 cells using siRNA specific to the human CyPA transcript to produce the HFsiCyPA and THP-1siCyPA cell lines. CyPA-specific or scrambled siRNA was stably transduced into HF or THP-1 cells using the pSilencer 5.1-U6 Retro vector (Ambion) following the manufacturer’s protocol. Stably transduced recombinant cells were analysed for the level of CyPA silencing using qRT-PCR. HF and THP-1 cells were treated with 0.5 μM CsA for 30 min prior to infection. CsA was added to the medium each time the medium was changed or the cells were washed.

**RT-PCR.** A PureLink RNA Midi kit (Invitrogen) was used to isolate RNA from HF and THP-1 mock-infected and infected cells following the manufacturer’s recommendations. SuperScript III reverse transcriptase (Invitrogen) was used to transcribe the cDNA following the manufacturer’s recommendations. Primers used to amplify the cDNA are listed in Table S1. Following amplification of the cDNA, products were loaded onto a 1 % agarose gel for analysis.
Real-time PCR. Total DNA was isolated from mock-infected and infected HF and THP-1 cells using a standard phenol/chloroform extraction method. Changes in viral DNA loads overtime were monitored using real-time PCR. Viral DNA levels were normalized to endogenous controls using primers against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β2-microglobulin (B2M) and CyPA (encoded by the PPAP gene) (endogenous controls), and UL123, UL54 and UL56 (viral primers) (Table S1).

qRT-PCR. Isolated RNA was transcribed into cDNA using SuperScript III reverse transcriptase. The cDNA was amplified in MicroAmp Optical 96-Well Reaction Plates using an Applied Biosystems Prism 7500 Fast Real-Time PCR System. To determine the level to which the CyPA transcript was silenced, gene expression was monitored using qRT-PCR with the primers syCyPA-F1/syCyPA-R1 and syCyPA-F2/syCyPA-R2, with GAPDH-F/GAPDH-R and B2M-F/B2M-R as endogenous controls (Table S1).

ACKNOWLEDGEMENTS

This project was supported by NIH grant HL63470.

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